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Viruses from each of the three major subgroups of human enteroviruses were tested for their stability in waters of various salinities. The major findings were: a) the most important factor influencing virus survival was water temperature with salinity having little, if any, effect; b) the individual viruses varied widely in their stability with Coxsackie B-5 being the most stable, ECHO virus 6 being intermediate, and poliovirus 1 the least stable; c) results of in situ studies indicated that the viruses were more labile in natural waters

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than in artificial seawater of the same salinity and that they were less stable in ocean water than in estuarine water; (2) the virucidal activity of seawater was associated with marine microorganisms.

Studies on the survival and distribution of pathogenic bacteria in estuarine and marine environments yielded the following results: a) large numbers of antibiotic-resistant bacteria were isolated from seawater off the Southeastern coast of the United States as well as from harbor and inshore waters; b) sediment samples, in general, contained fewer antibiotic-resistant bacteria strains than seawater samples; c) antibiotic-resistant strains of coliforms and Vibrio spp. were isolated from the holding tank of an ocean going vessel at sea; and d) heavy metal and multiple-drug resistant bacteria were isolated from the Chesapeake Bay and from the Puerto Rico Trench. Plasmids associated with these resistant bacteria were characterized by molecular genetic techniques. The curing of several strains of bacteria of plasmids resulted in a concomitant loss of resistance, indicating that the resistance is plasmid-mediated.

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FINAL REPORT

(UM/ONR 6)

Survival of Microbial Pathogens in the Marine Environment

by

R. R. Colwell and F. M. Hetrick

Department of Microbiology
University of Maryland
College Park, Maryland 20742

1 May 1979

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SUMMARY OF RESEARCH ACCOMPLISHED

(A) Virus Survival Studies

1. Effect of temperature and salinity on enterovirus stability.

Viruses from each of the three major subgroups of enteroviruses were used in this study: poliovirus type 1, coxsackievirus B-5, and Echovirus 6. Synthetic seawater was adjusted to 10, 20, and 34 parts per thousand and the temperatures examined were 4, 15, 25, and 37 C. As expected, the most important factor influencing virus survival time was the water temperature in that the higher the temperature the more rapid was the loss of viral infectivity. Of the three enteroviruses tested, coxsackievirus B-5 was the most stable with Echovirus 6 being intermediate and poliovirus 1 the least stable. These results are probably representative of the diversity in stability one would find if all the 70+ human enteroviruses were studied. Water salinity had very little, if any, effect on the stability of enteroviruses.

It is also apparent from these studies that enteric viruses can survive for relatively long periods of time in estuarine and marine salinities when the water temperature is 15 C or lower. For example, infectious poliovirus was still detectable after 20 weeks at 15 C and for 40 weeks at 4 C; Echovirus 6 was present after 24 weeks at 15 C and for 40 weeks at 4 C while coxsackievirus B-5 was detectable for 53 weeks, the last sampling time, at both temperatures.

2. Enterovirus survival studies conducted in situ.

These studies were conducted at the University of Delaware Marine Laboratory at Lewes and at the Chesapeake Biological Laboratory at Solomons, Md. Dialysis bags containing the 3 enteroviruses were immersed into plastic tanks continuously charged with free-flowing marine (salinity of 28-30 ppt) or estuarine (salinity of 8-12 ppt.) water.

As in the previous studies, water temperature proved to be the important factor as the viruses were more labile during the summer months than during the winter months. Similarly the relative stabilities of the agents studied were coxsackievirus B-5, Echovirus 6 and poliovirus 1 in decreasing order. In ocean water all the poliovirus infectivity was lost by 27 days during the summer and by 65 days during the winter months while the Echovirus survived slightly longer i.e., 31 days in the summer and 70 days in the winter. It took 7 weeks for total inactivation of the coxsackievirus in the summer, however, only a 2 log drop in infectivity occurred over an 80 day period in the winter. In the estuarine water, similar results were obtained with the coxsackievirus retaining considerable infectivity after 116 days of incubation during the winter time.

Although the enteroviruses again proved to be quite stable, results of these in situ studies indicated that they are more labile in natural waters than in the artificial seawater and that the viruses are less stable in ocean water than in estuarine water

3. Enterovirus survival studies in untreated seawater.

These controlled laboratory studies showed that enteroviruses are indeed more labile in untreated natural seawater than in synthetic seawater of the same salinity held at the same temperature. For example, we

previously reported that at 4 C, polio, ECHO-6, and coxsackie B-5 viruses survived for 40, 46, and 53 weeks respectively in synthetic seawater whereas in natural seawater at the same temperature their survival times were 28, 28, and 76 days respectively. Similar findings were made at incubation temperatures of 15 and 25 C in that the viruses were inactivated more rapidly in untreated seawater.

Parvoviruses are among the hardiest of the animal viruses with stability characteristics very similar to those of the hepatitis viruses. One of the human parvoviruses, H-1 virus, was selected as being representative of the group and its stability in untreated ocean water determined. As observed in our previous studies with enteroviruses, a decrease in survival time was seen with increasing water temperatures. However, H-1 virus proved to be more stable than any of the enteroviruses previously tested in that little or no drop in infectivity occurred at 4 or 15 C during 48 weeks of incubation. At 37 and 25 C, viral infectivity was lost with 10 and 14 weeks respectively, however, all 3 enteroviruses were inactivated within a week at 37 C and within 7-10 weeks at 25 C.

Although peripheral to the main objectives of the contract work, studies were initiated to determine if human lymphocyte cell cultures might present advantages in the parvovirus studies since the standard monolayer assay for these viruses is time-consuming. Two publications resulted from this work and ONR support is acknowledged in both. One describes the productive infection of a human B cell line with several parvoviruses while the other describes the establishment of a persistently infected lymphocyte culture system.

4. Inactivation of the enteroviruses in seawater at pasteurization temperatures.

One possible onboard sewage treatment process that the Navy could use would be heating of the effluent prior to discharge to thermally inactivate viral and bacterial pathogens. In order to provide some baseline data in this regard, we determined the rate of virus inactivation in various salinity waters when exposed to pasteurization (62 C) temperatures. All these viruses were totally inactivated (initial inoculum contained 10^6 pfus/ml) within 30 minutes at 62 C. One interesting finding is that the rate of virus inactivation was slowest at 34 ppt salinity as compared to the two other salinities. It is known that divalent cations stabilize enteroviruses to heating and this is the most probable explanation for this finding.

5. Studies on the virucidal activity of seawater.

The virucidal activity of seawater was eliminated or greatly reduced by either autoclaving the water sample or by passage through a 0.45 micron membrane filter. The activity could be restored to either autoclaved or filtered water by eluting the material retained by the filter and adding it back to the water. These results indicated that marine microorganisms or their metabolic products may be responsible for the virus inactivation.

Marine bacteria were isolated from samples of ocean water possessing virucidal activity. One isolate was a gram-negative, rod-shaped bacterium which was subsequently identified as Pseudomonas atlantica. This organism was found to possess virucidal activity against poliovirus types 1 and 3

but not against type 2. Culture filtrates of this organism did not have any virucidal activity indicating that it was associated with the bacterial cell. Formaldehyde treatment of P. atlantica greatly diminished its virus inactivating activity.

Radioisotope-labeled (^{3}HdU) poliovirus 1 became associated with P. atlantica as demonstrated by the increased radioactivity of the bacterial cells trapped on a membrane filter. Adsorption of the virus to the bacterial cells is also suggested by other experiments which showed increased radioactivity with untreated bacteria at the same time virus inactivation was taking place. Hg-treated P. atlantica retain the virus-inactivating capacity but they do not retain the radioactive label as do untreated bacteria. This suggests in the case of untreated P. atlantica cells, a process of adsorption of poliovirus 1 to the bacteria, subsequent inactivation of the virus, and retention of the altered virus, or at least the labeled RNA of the virus, by the bacteria.

6. Drug Resistant Bacteria.

Studies on the survival and distribution of pathogenic bacteria in the estuarine and marine environment were undertaken. Antibiotic resistant bacteria were isolated from seawater samples collected in the Atlantic Ocean off the Southeastern Coast of the United States. Large numbers of antibiotic-resistant bacterial strains were found in harbor and inshore waters. However, the percentage of antibiotic-resistant bacterial strains was higher in several seawater samples

collected off-shore than near shore. Bacteria resistant to tetracycline, chloramphenicol, and streptomycin were found in nearly all samples collected, including those from 200 miles off-shore and at depths to 8,200 m. Sediment samples, in general, were found to contain smaller populations of antibiotic-resistant bacterial strains, compared with the seawater samples examined. Antibiotic-resistant bacteria exhibiting phenetic characteristics common to autochthonous marine bacteria were examined in detail and several of the isolates exhibited unstable resistance, with antibiotic resistance transferable to recipient Escherichia coli cells. Deoxyribonucleic acid preparations from ten strains were examined using ethidium bromide-cesium chloride gradients. Six of the strains were found to contain covalently closed circular plasmid DNA.

Antibiotic resistant strains of coliforms and salt-requiring bacteria were isolated from the holding tank of an ocean-going vessel at sea. Plasmid-mediated antibiotic resistance was discovered in four strains of Vibrio spp. isolated from water samples collected from the tank.

Heavy metal and multiple-drug resistant bacteria, the resistance of which was mediated by plasmids, were isolated from Chesapeake Bay and from the Puerto Rico Trench of the Atlantic Ocean. Plasmids associated with heavy metal resistance and drug resistance in these strains were characterized by molecular genetic techniques. Curing of several of the strains was achieved, with concomitant loss of resistance,

providing conclusive evidence of the mediation of resistance via plasmids. It is hypothesized, from results of research accomplished under this contract, that in areas of heavy metal contamination in the marine environment, selection for multiple drug resistance will occur, in part due to the linkage of genes conferring heavy metal and drug resistance and to mediation of these genes via plasmids, with plasmid transfer able to occur in the natural environment.

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